

Paediatric Reference Values for Urinary Catecholamine Metabolites Evaluated by High Performance Liquid Chromatography and Electrochemical Detection¹⁾

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Summary: The majority of the published reference range data on catecholamines excretion by healthy children is incomplete and often contradictory (1). We assayed in the urines of 127 healthy children the values of the catecholamines (norepinephrine, epinephrine, dopamine) and their methylated metabolites (normetanephrine, metanephrine, 3-methoxytyramine) for the determination of paediatric reference ranges. Data were expressed as µg/24 h, µmol/24 h and mmol/mol creatinine. An isocratic HPLC procedure by ion-pair reversed phase chromatography on a C18 column, using a single mobile phase containing formic acid, acetonitrile, diethylamine and octane sulphonic acid (ion pairing agent), permitted the separate assay of the various fractions of total catecholamines. The relations between each biogenic amine and age were studied and reference values were determined as a function of age.

Introduction

Neural crest tumours such as neuroblastoma and ganglioneuroma are associated with an abnormal secretion of catecholamines in tissue and body fluids. Assays of urinary vanillylmandelic acid, homovanillic acid, and dopamine permit biochemical diagnosis of neuroblastoma in approximately 80% of patients (2).

Differential diagnosis of pheochromocytoma, neuroblastoma and related diseases requires multiple investigations, among which the determination of the catecholamines and of their metabolites is of primary importance (3).

Catecholamines are compounds containing aliphatic amines attached to a benzene ring bearing two hydroxyl groups in 3,4 position (catechol). They are derivatives of the amino acid tyrosine. Catecholamines are extensively metabolized; only 2–10% are eliminated in urine, the major part in conjugated form, and less than 20% are eliminated as methylated metabolites (essentially conjugated): normetanephrine, metanephrine and 3-methoxytyramine. Norepinephrine is the main neurotransmitter of the autonomic nervous system, dopamine is both a precursor of norepinephrine and a neuromediator of the central nervous system. The adrenal medulla contains norepinephrine N-methyltransferase, which converts norepinephrine into epinephrine. The catecholamine metabolic pathways are illustrated in figure 1.

High-performance liquid chromatography with amperometric detector (HPLC-EC) methods allows the separate assay of various catecholamine fractions: norepinephrine, epinephrine, dopamine, normetanephrine, metanephrine, 3-methoxytyramine (4).

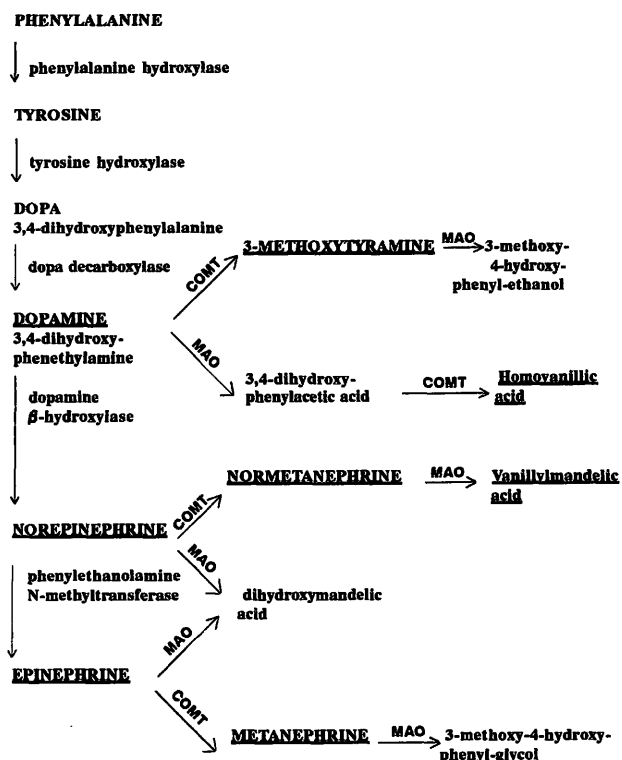


Fig. 1 Catecholamine pathways. MAO: monoamine oxidase; COMT: catechol-O-methyl transferase [Modified from Candito M. et al. (3)] The underlined compounds are those assayed in our laboratory.

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Materials and Methods

Sample collection

Twenty-four-hour urine specimens were collected in polyethylene boxes containing 4 ml per litre of an acidic buffer mixture of CH_3COOH 4 mol/l/ NaOH 4 mol/l (4 + 1, by vol.) as preservative. The volume of the specimen was measured; an aliquot was stored at -20°C . Normal reference values were calculated on 127 healthy children aged 21 days to 14 years (mean: 50 months, median: 36 months): some of them were from the school and some were outside or inside patients without specific symptoms of hypertension. All the above-mentioned compounds were assayed in the urine of children before any treatment.

Analytical methods

Creatinine values were measured by the *Jaffe* method (5) on the Synchron CX 7 (Beckman Instruments, Inc. Brea, CA 92621-6209).

For the determination of total (free and conjugated) concentrations of catecholamines, the urine samples were hydrolyzed. To 1 ml urine and 250 μl of internal standard (dihydroxybenzylamine) HCl concentrated to pH 1 was added and then heated to 100°C in a closed tube for 20 minutes (6). After hydrolysis, the pH of the mixture was adjusted to 6.5 with 2 mol/l and 0.5 mol/l NaOH successively. The urine neutralised was purified by a cation exchange column of resin Amberlite CG 50 (Acros Organics, New Jersey, USA); the biogenic amines were eluted with 5 ml of 0.1 mol/l nitric acid. The eluate (20–50 μl) was injected into the HPLC system (4, 6).

HPLC-EC was performed on a 125 mm \times 4 mm i. d. LichroCART-Superspher 100 RP-18 (E. Merck, Darmstadt, Germany) column filled with 5 μm particle size. A similar pre column (LiChrocart 4-4) was used.

The system was automated with an autosampler model ISS 100 (Perkin Elmer Corporation, Norwalk, USA). The results were processed by a computing integrator model LC100 (Perkin Elmer).

Mobile phase: Formic acid (100 nmol/l)/citric acid (1 mmol/l)/octane sulphonic acid (0.4 mmol/l)/ethylenediaminetetraacetic acid (EDTA) (0.1 nmol/l) and 5% acetonitrile, diethylamine (0.25%), pH 3.1 with KOH 0.5 mol/l.

Flow rate: 0.7 ml/min isocratic

Detection: 750 mV by model LC 4C electrochemical detector (BAS, Indiana, USA).

Statistical methods

The correlation among biogenic amines and between each catecholamine and age was investigated by non-parametric *Spearman* test.

When the population had a *Gaussian* distribution, reference limits were determined by a linear regression as a function of the childrens age.

If data distribution was not normal, the different groups were compared by *Kruskal-Wallis* test.

Results

Analytical results

Intra- and inter-assay imprecisions were determined from analysis of Ortho and Biorad normal and pathological control urines and from a normal urine.

Intra-assay coefficients of variation (CV) were: 10.5% for norepinephrine, 9.5% for epinephrine, 6% for dopamine, 7% for metanephrine, 8.5% for normetanephrine

and 5% for 3-methoxytyramine. Inter-assay CVs were: 13.5% for norepinephrine, 10.5% for epinephrine, 5% for dopamine and 3-methoxytyramine, 6% for metanephrine and normetanephrine.

Recoveries were different: 90% for norepinephrine, 89% for epinephrine, metanephrine and normetanephrine, 102% for dopamine and 92% for 3-methoxytyramine. We had a good linearity from 50 pg to 100 ng of norepinephrine with less than 5% deviation from the fit line.

The determination of the lower detection limits of the components was based on the linear dilution of their standards. The detection limits were 9 nmol/l for norepinephrine, 11.5 nmol/l for epinephrine, 11.9 nmol/l for dopamine, 11 nmol/l for metanephrine and 11.3 nmol/l for normetanephrine and 7.1 nmol/l for 3-methoxytyramine, using 50 μl injection volume of a standard solution and of an Ortho normal control urine appropriately diluted.

Statistical results

Different catecholamine metabolites were poorly correlated among themselves: this event demonstrated a different information provided by each analyte.

All biogenic amine levels, except normetanephrine, were strictly related to age (r = about 0.7). Therefore it was necessary to express reference values in relation with patient age.

If the values were calculated as $\mu\text{g}/24\text{ h}$ or $\mu\text{mol}/24\text{ h}$, they appeared with no *Gaussian* distribution even after logarithmic transformation. On the contrary these values showed a normal distribution if they were expressed as mmol/mol creatinine and transformed in natural logarithm.

Opportune age intervals that appeared significantly different were determined for epinephrine, norepinephrine, dopamine, normetanephrine, metanephrine, and 3-methoxytyramine levels calculated as $\mu\text{g}/24\text{ h}$ and $\mu\text{mol}/24\text{ h}$. These intervals are graphically illustrated in figure 2.

Table 1 reports upper reference limits and their confidence intervals.

The relations between childrens age and the different catecholamine metabolites expressed as mmol/mol creatinine are reported in figure 3 as linear regressions. Their upper reference limits are also graphically illustrated in figure 3.

Discussion

The analytical method appeared easy and reproducible.

These reference ranges were established in agreement with International Federation Clinical Chemistry (IFCC)

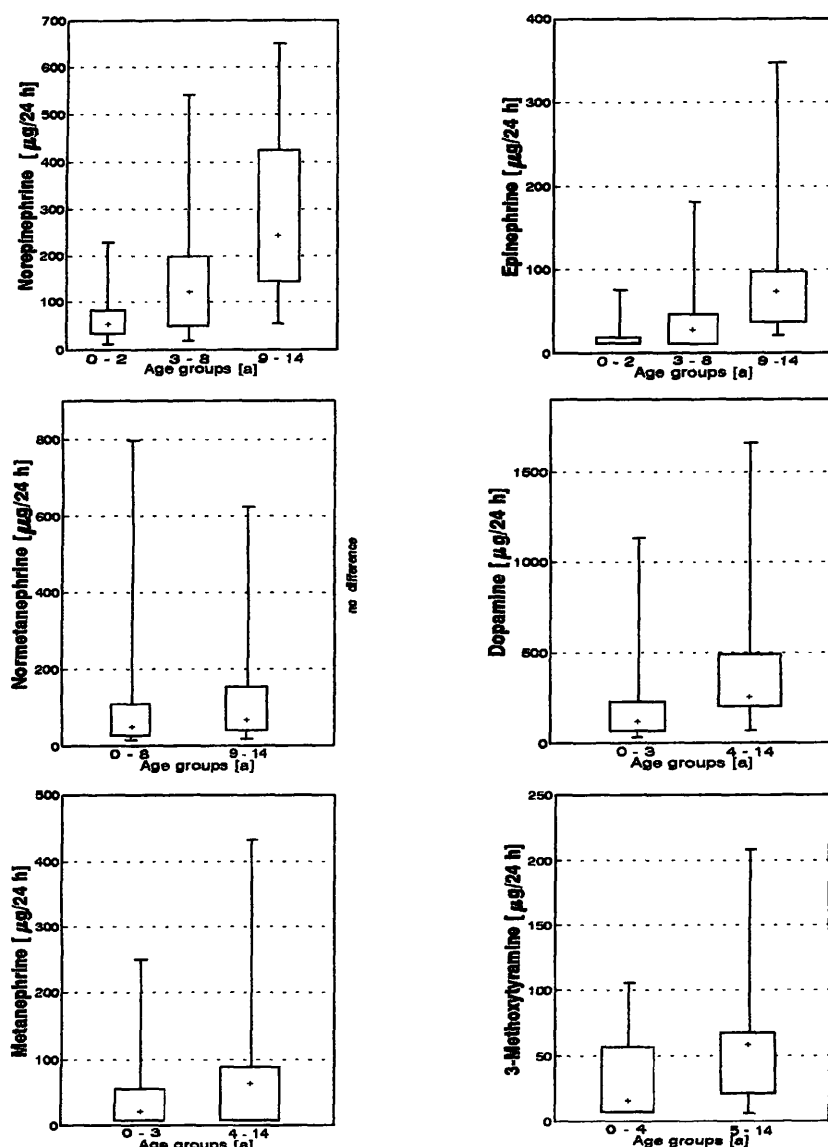


Fig. 2 Differences among distributions.

Tab. 1 Reference limits of catecholamine urinary metabolites.

	Age groups (a)	Catecholamine		Catecholamine	
		Concentration	Confidence intervals	Concentration	Confidence intervals
		($\mu\text{g}/24\text{ h}$)	($\mu\text{g}/24\text{ h}$)	($\mu\text{mol}/24\text{ h}$)	($\mu\text{mol}/24\text{ h}$)
Norepinephrine	0-2	<274	247-301	<1.62	1.46-1.78
	3-8	<590	579-601	<3.50	3.42-3.55
	9-14	<680	519-841	<4.02	3.07-4.97
Epinephrine	0-2	<38	35-41	<0.21	0.19-0.22
	3-8	<150	139-161	<0.82	0.76-0.88
	9-14	<360	291-428	<1.97	1.59-2.34
Normetanephrine	0-14	<827	326-878	<4.52	1.78-4.79
Dopamine	0-3	<1300	1240-1360	<8.49	8.10-8.88
	4-14	<1876	1663-1989	<12.25	10.89-12.98
Metanephrine	0-3	<254	238-270	<1.29	1.21-1.37
	4-14	<461	433-489	<2.34	2.19-2.48
3-Methoxytyramine	0-4	<103	94-112	<0.62	0.56-0.67
	5-14	<210	192-228	<1.26	1.15-1.36

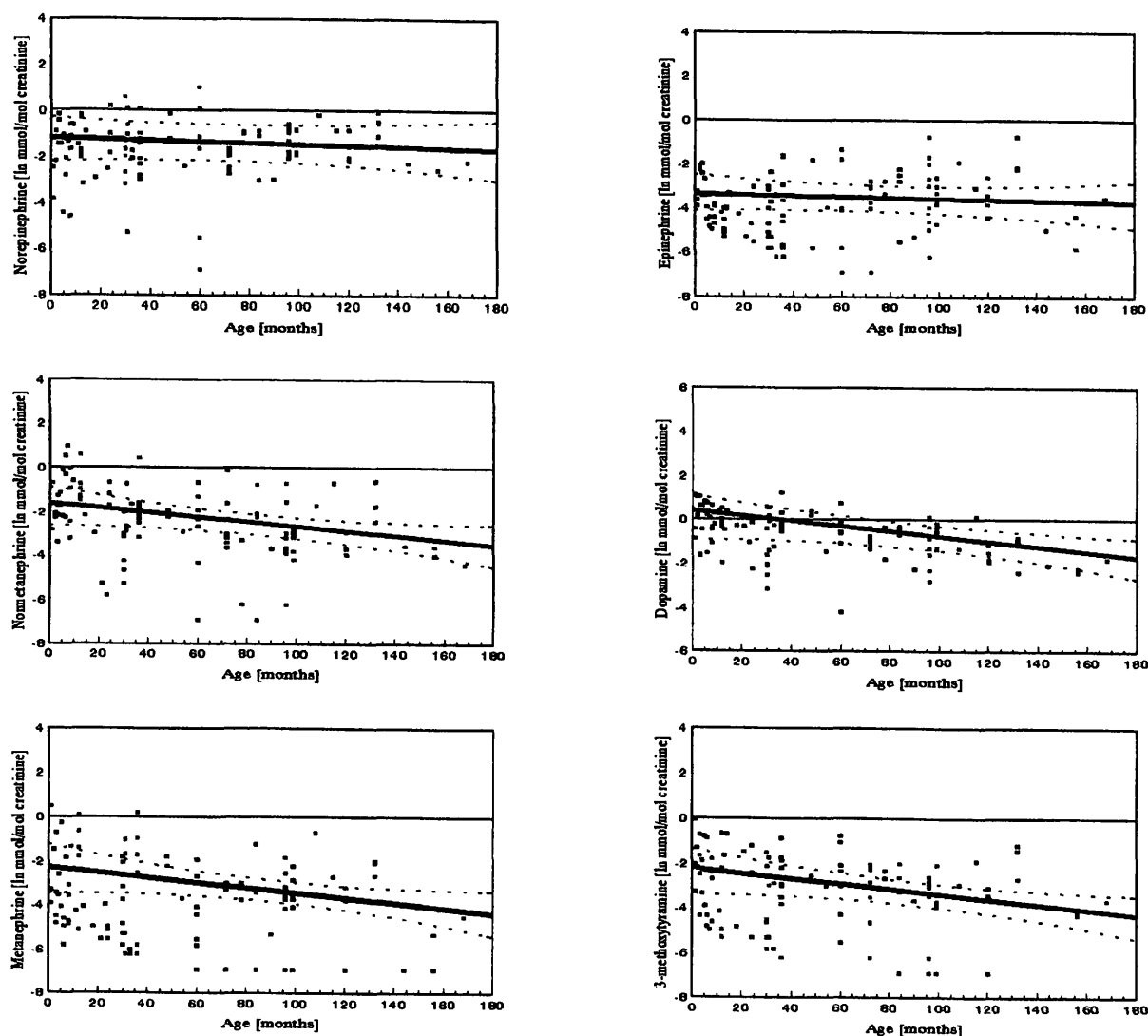


Fig. 3 Linear regressions.

$\ln \text{Norepinephrine} = -1.35 - 1.34 \times \text{age (months)}$
 $\ln \text{Epinephrine} = -3.28 - 0.00007 \times \text{age (months)}$
 $\ln \text{Normetanephrine} = -1.36 - 1.52 \times \text{age (months)}$

$\ln \text{Dopamine} = -0.0051 - 0.001 \times \text{age (months)}$
 $\ln \text{Metanephrine} = -2.71 - 0.012 \times \text{age (months)}$
 $\ln \text{3-Methoxytyramine} = -2.23 - 0.0011 \times \text{age (months)}$

recommendations (7) and the data were described with an adequate graphic, preceded and complemented by a formal statistical analysis (8). We carefully selected local healthy children among our population.

Our reference values, with the exception of 3-methoxytyramine, are higher than those reported in the literature (1, 3); the increase of catecholamine excretion with age was already reported (9).

Many factors influence the large variation in published paediatric reference ranges for urine metabolites of catecholamines (1). There is no uniformity in the units reported from different laboratories. Data were expressed as mmol/mol creatinine, mmol/day or mmol/kg body weight with no possibility of comparing results (10–12).

We chose to evaluate the total catecholamines (free and conjugated) with a published method (6) improving only

the detection of these compounds with the use of an amperometric detector (4).

We assayed the total catecholamines because this way the information on the metabolism is more complete and the data give better information about the lowest variation of the excretion of the tumour (3). We had a good correspondence with the clinical findings of the patients affected by neuroblastoma and also of the children with hypertension.

The levels related with the creatinine excretion must be evaluated carefully because in the literature (13) a lot of articles describe the possible interference from diet (ingestion of meat) (14), drugs (15, 16), analytical variation (5–7%) and intra-personal fluctuation for younger children (13). All borderline values should be repeated routinely.

Conclusions

Quantification of total urinary catecholamines is a reliable means of diagnosis and monitoring of neuroblastoma in children. None of these tests are 100% sensitive or specific in tumour detection and all metabolites should be measured. Catecholamine assays on serial urine samples of previously diagnosed patients proved to be helpful as prognostic markers, to identify relapse and response to chemotherapy.

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